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Molecular Basis for Rh_{null} Syndrome: Identification of Three New Missense Mutations in the Rh50 Glycoprotein Gene

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Rh_{null} is a rare autosomal recessive disorder characterized by an absence of Rh antigens and a varying degree of hemolytic anemia and spherostomatocytosis. We report studies of two Japanese Rh_{null} cases and describe three new missense mutations of *RHAG,* the locus that encodes Rh50 glycoprotein and modulates Rh antigen expression. In Rh_{null}(HT), *RHAG* harbored in exon 6 two G→A transitions, GTT→ATT and GGA→AGA, which cause Val^{270}→Ile and Gly^{280}→Arg substitutions, respectively. These missense mutations were cotransmitted from the propositus to the children and were predicted to reside in endoloop 5 and transmembrane (TM) segment 9, respectively. In Rh_{null}(WO), *RHAG* contained in exon 9 a single G→T transversion, GGT→GTT, which caused a Gly^{380}→Val missense change in TM12 segment. The G→T transversion, which is located at the +1 position of exon 9, had also affected pre-mRNA splicing and caused partial exon skipping. Although both Rh_{null} cases had a structurally normal *RH* antigen locus, hemagglutination and immunoblotting showed no expression of Rh antigens or proteins. These results correlate each mutation with a structural defect in the respective TM domain of Rh50 glycoprotein. Am. J. Hematol. 62:25–32, 1999. © 1999 Wiley-Liss, Inc.

Key words: Rh deficiency syndrome; suppressor mutations; membrane proteins; erythrocytes; genetic defect

INTRODUCTION

Rh_{null} disease is characterized by the lack of Rh blood group antigens in association with a varying degree of chronic hemolytic anemia and spherostomatocytosis [1]. It arises by two distinct genetic mechanisms: the amorph type is caused by mutations at the Rh antigen locus itself, whereas the regulator type is caused by mutations at a separate suppressor modulating Rh expression [2]. Recent studies identified in Rh_{null} individuals a heterogeneous spectrum of mutations in *RH* and *RHAG* genes encoding Rh30 polypeptides and Rh50 glycoprotein, respectively [3–8]. These results show that functional *RH* and *RHAG* loci both are essential for Rh antigen expression and for maintenance of the integrity of the erythrocyte membrane. Most Rh_{null} cases are attributed to the occurrence of a defective regulator [1,2] whose identity has been established to be *RHAG* or Rh50 gene [9]. Of the regulator defects reported to date, two are point mutations leading to missense amino acid substitutions [3,7,8], whereas others are either small exonic deletions or splice site mutations resulting in frameshift and premature termination [3,6,7]. The two missense changes in Rh50 glycoprotein, Ser79Asn and Gly279Glu, have been found in Rh_{mod}(VL) [3] and Rh_{null}(YT) [7,8], respectively. We report here molecular genetic studies of two

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TABLE I. Synthetic Oligonucleotides for Analysis of Rh50 Glycoprotein Gene

Category[a]	Sequence	nt position[b]	Location
I. cDNA PCR			
3′-UTa	5′-AATGGGAAAGGAAGCTGGAGAGCA-3′	1321–1298	3′-UTR
Ex-1s	5′-AGTGTGCCTCTGTCCTTTGCCACA-3′	−27–−4	exon 1
Ex-4s	5′-GAAGAGTCCGCATACTACTCAGAC-3′	601–624	exon 4
Ex-5a	5′-CTGTTTGTCTCCAGGTTCAGCAAT-3′	708–605	exon 5
Ex-10a	5′-CCATGTCCATGGAACTGATTGTCA-3′	1256–1233	exon 10
$(dT)_{16}$ anchor	5′-GTCATGACTCGAGTCGACATCGA$(T)_{16}$-3′		
II. 3′ RACE			
Ex-5s	5′-AGCTTTAACTCGGCCATTGCTGAA-3′	670–693	exon 5
Ex-7s	5′-GATACATGTGGGGTCCATAACCTC-3′	976–999	exon 7
3′-UTa	as above		
II. Genomic PCR			
In-5s	5′-TTTCAACTGAAGAATGTGATGTGG-3′	85–107	intron 5
Ex-6a	5′-AGTCAGGAACTTGTATCCAAGCAC-3′	945–922	exon 6
In-6a	5′-CCAAAAAGAAGGCAAATGGTCTCC-3′	111–134	intron 6
In-8s	5′-TGTTCTAAACTTCTGGCAGTAAAG-3′	111–134	intron 8
Ex-9a	5′-ACCTTCCAATAAACAGAATCATCA-3′	1334–1191	exon 9
In-9a	5′-CCTACCATGCCAGGCTAACTCAAT-3′	101–124	intron 9

[a]Sense (s) and antisense (a) primers to 3′-UTR, exon (Ex), and intron (In) are denoted.
[b]Nucleotide positions of exon primers are accounted from the first base of initiation codon ATG, whereas intron primers are designated by their distance from the exons they encompass.

Japanese Rh_{null} cases and describe three new point mutations in *RHAG* causing missense amino acid substitutions. Intriguingly, the Val270Ile change is also seen in the Rh homolog from *C. elegans*, whereas the other two target glycines (Gly280Arg and Gly380Val), the two residues conserved in the 9th or 12th transmembrane (TM) domain of Rh50 proteins. Of the two nucleotide (nt) changes (808A and 1139T) located at the +1 position of exons, 1139T also has caused aberrant pre-mRNA splicing by partially inactivating its adjacent acceptor splice site.

MATERIALS AND METHODS

Blood Samples, Hemagglutination and Immunoblot Analysis

Blood samples used as controls were from D+ (genotype, *DCe/DCe*) and D- (genotype, *dce/dce*) donors. Rh_{null} samples were from two unrelated Japanese: HT and WO. The heterozygous children (RT and YT) of HT were also studied, but family members of WO were not available. Hemagglutination was performed using standard methods. Isolation of membranes and immunoblot analysis were as described [5–7]. The monoclonal antibodies used were LOR-15C9 against RhD [10] and 2D10 against Rh50 [11]. To visualize protein bands, peroxidase-conjugated anti-human Ig and anti-mouse Ig were used as second antibodies for LOR-15C9 and 2D10, respectively.

Southern Blot Analysis

Genomic DNA was isolated from leukocyte pellets. The Rh50 probe was a full-length cDNA isolated from a placenta cDNA library T[6], this cDNA spans nt -6 to 1256 and codes for 409 amino acids of Rh50. Rh30 cDNA probes span the 5′ (exon 1–3, nt 1–480), middle (exon 4–7, nt 515–1073) and 3′ portions (exon 8–10 plus 3′-untranslated region or UTR, nt1074–1456), respectively [12]. Southern blots were hybridized with random primer-labeled ^{32}P-probes.

Reverse Transcriptase-Polymerase Chain Reaction (RT-PCR)

Total RNA was isolated from reticulocyte hemolysates [13], and extracted with the Trizol reagent (GIBCO-BRL, Gaithersburg, MD). Analysis of Rh50 and Rh30 transcripts by RT-PCR was performed as previously described [6,12]. Table I shows the primers used. Approximately 2 μg of total RNA was annealed with a 3′-UT primer or an anchored oligo$(dT)_{16}$ primer [14]. Single-stranded cDNA synthesis was carried out in a 20 μl reaction volume, incubated at 42°C for 75 min and inactivated at 72°C for 10 min 2 μl of cDNA solution was taken and amplified with two primers specific for the Rh50 or Rh30 gene. Amplification was repeated 35 cycles at 94°C for 1min, 55°C for 45 s, and 72°C for 1 min. The last step of chain extension was at 72°C for 10 min.

Amplification and Analysis of *RH* and *RHAG* Genomic Sequences

To determine the genotype and verify the missense changes, the *RH* and *RHAG* sequences were amplified by using total genomic DNA as a template. Exon PCR assays by either RFLPs or genomic sequencing were as

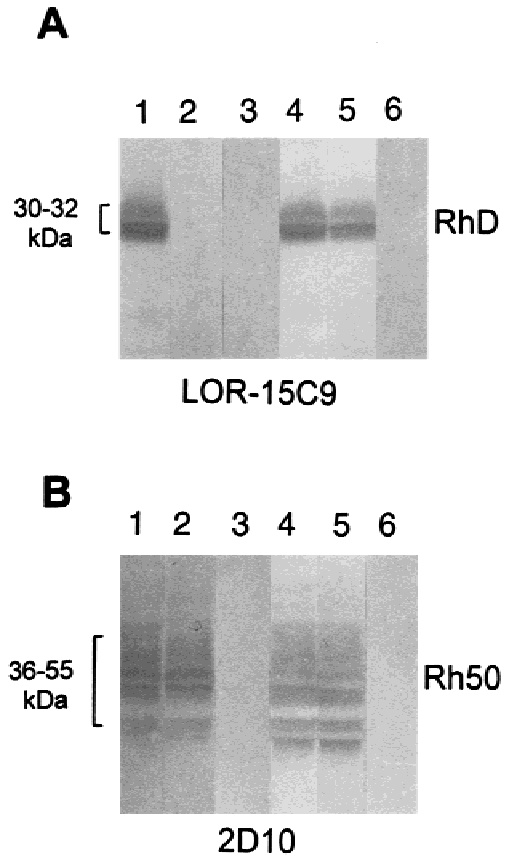

Fig. 1. Immunoblotting analysis of RBC membrane Rh30 and Rh50 proteins. A: Immunoblots were probed with the monoclonal antobody LOR-15C9 against RhD. Lane 1, D+ control; lane 2, D- control; lane 3, Rh_{null} (HT); lanes 4 and 5, D+ children of HT (RT and YT); and lane 6, Rh_{null} (WO). A band of 30–32 kDa is seen in D+ persons. Like D-, the two Rh_{null} probands lack reaction with this antibody, although they each have a normal *RHD* gene (see Fig. 2). The membrane proteins were electrophoresed in polyacrylamide gels on nonreducing conditions. B: Immunoblots of membrane proteins probed with the monoclonal antibody 2D10 against Rh50. Lanes are the same as in A. A broad diffuse band in the range of 36–50 kDa is seen in controls and HT's children but not in Rh_{null} propositi.

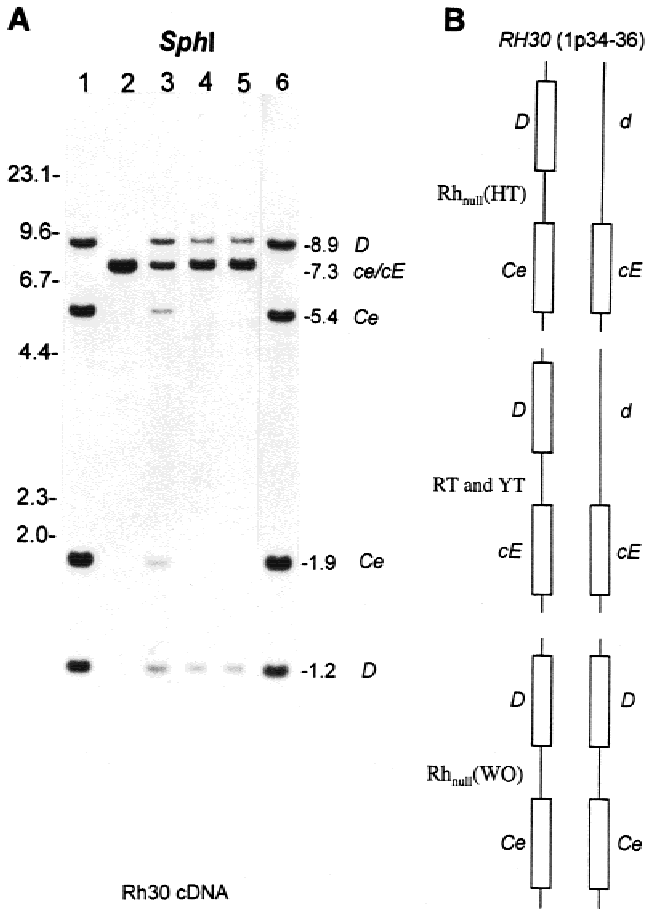

Fig. 2. Southern blot analysis of *RH* locus. A: Genomic DNA was digested with restriction enzyme *Sph*I, and the blot was probed with the Rh30 cDNA encompassing exons 4–7. Lanes are designated as in Figure 1. The lambda phage DNA cleaved with *Hind*III was used as markers whose size in kb is shown at left. The size and gene origin of various bands are indicated at right. Note that there is no gross deletion or rearrangement of *RH* in Rh_{null}(WO). However, the *RHD*-specific 8.9 and 1.2-kb bands show reduced intensity in Rh_{null}(HT) and the children (RT and YT), indicating a deletion of *RHD* in one haploid genome. B: Genotypic status of the *RH* locus, as deduced from Southern blots and confirmed by transcript analysis. *RHD* and *RHCE* (i.e., *Ce* and *cE*) are denoted by two vertical bars and the deletion of *RHD* by *d*. Rh_{null}(HT) was genotyped *DCe/dcE* and the children genotyped *DcE/dcE*. The genotype of Rh_{null}(WO) was *DCe/DCe*.

previously described [12]. Table I lists the synthetic primers for PCR. The amplified *RHAG* exon 6 and exon 9 products were purified and subjected to direct sequencing and/or electrophoretic analysis by single-strand conformation polymorphism (SSCP).

Subcloning and Nucleotide Sequencing

The cDNA and genomic products were purified by 5% polyacrylamide gel electrophoresis. The eluted products were either directly sequenced or sequenced after subcloning. In the latter case, the DNA was ligated to the TA cloning vector pCR2.1 according to the supplier's specifications (Invitrogene, San Diego, CA). Sequences were determined on an automated DNA sequencer using fluorescent dye-tagged chain terminators (Applied Biosystem, Foster City, CA).

RESULTS

Blood Typing and Immunoblot Analysis

The parents of Rh_{null}(HT) were consanguineous, but no such information was known for Rh_{null}(WO). Typing of TH showed a negative reaction with antibodies for D, C, c, E, e and Rh17 antigens. HT's children typed D + C-c + E + e-. To clarify the e antigen status, we tested HT's RBCs with seven monoclonal anti-e and one poly-

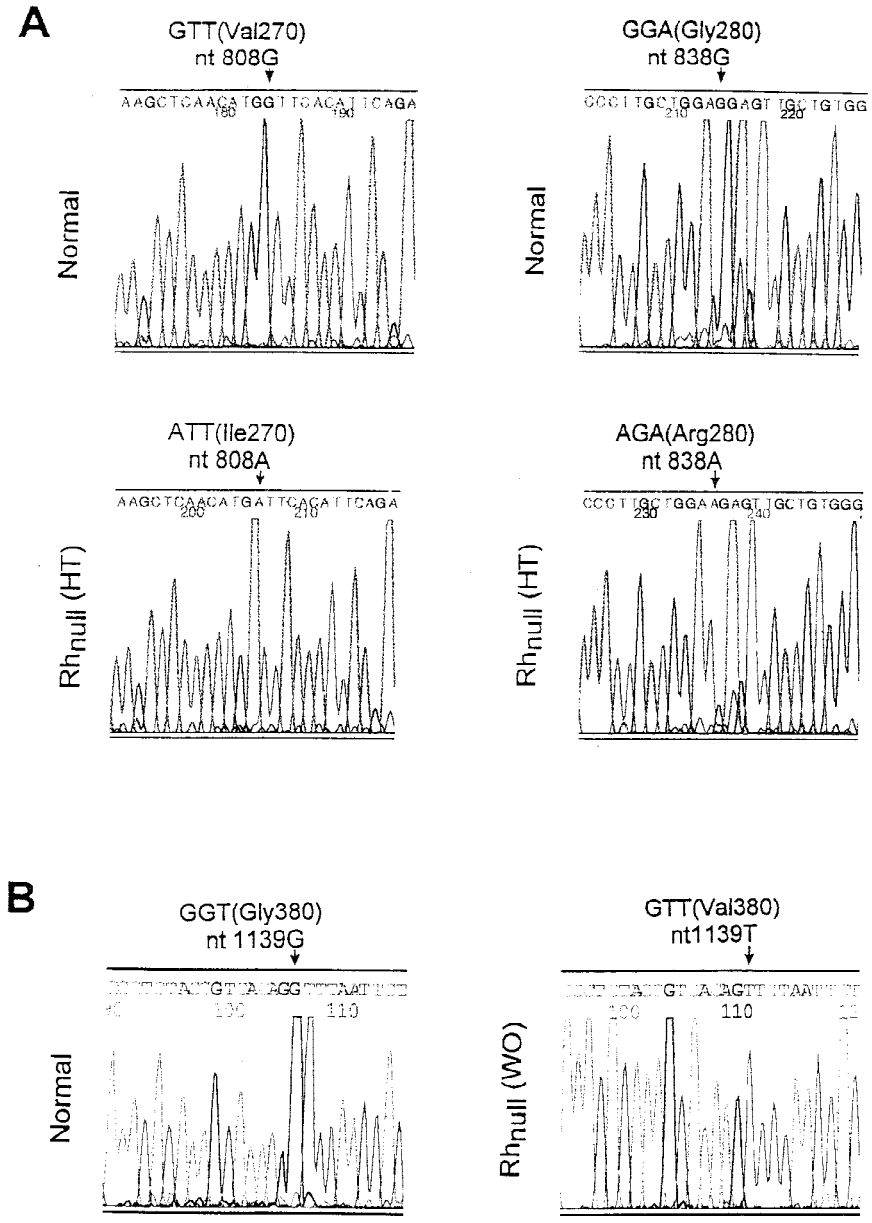

Fig. 3. Identification of three novel point mutations in the Rh50 transcript by cDNA sequencing. The Rh50 cDNA was reverse-transcribed using either a gene-specific 3′-UT primer or an anchored oligo(dT)16 primer and then amplified with two pairs of upstream primers (Table I). The region encoding a full-length protein of 409 amino acids was sequenced to completion. A: The sequence profile showing double G→A transitions at nt 808 and 838 of exon 6 in Rh_{null}(HT). B: The sequence profile showing a single G→T transversion at nt 1139 of exon 9 in Rh_{null}(WO). In each case, the a normal sequence from a *DCe/DCe* homozygote is shown for comparison. The amino acids specified by the wild-type and missense codons are denoted.

clonal anti-e as a control. All anti-e were nonreactive whether the cells were treated or untreated with papain. Hemagglutination test was also performed for Rh-associated proteins, including Rh50, LW, CD47, and GPB. In both Rh_{null} propositi, Rh50 and LW were absent, CD47 was weakly expressed, and the GPB-borne SsU antigens were variable. To further investigate the expression of carrier proteins, immunoblot analysis of Rh proteins was done with antibodies LOR-15C9 and 2D10. Tests with LOR-15C9 showed an absence of the RhD protein in all but D+ lanes (Fig. 1A). A broad diffuse band, probably representing differentially glycosylated forms of Rh50, was seen in both D+ and D- but not in Rh_{null} lanes (Fig. 1B). The results established the null status of Rh phenotypes in HT and WO.

Structure and Expression of Rh30 Genes

To rule out the amorph type for the two Rh_{null} cases, the Rh30 genes were characterized. Fig. 2 shows a typical blot hybridized with the cDNA covering exons 4–7, the polymorphic region of *RH* [15]. The intensity of *RHD*-specific 8.9 and 1.2 kb bands was reduced in Rh_{null}(HT) and the children, but no gross change was seen in Rh_{null}(WO)(Fig. 2A). As shown by *Sph*I RFLPs, Rh_{null}(HT) had a *DCe/dcE* genotype lacking *RHD* in one chromosome. However, Rh_{null}(WO) was identical with the D+ control, having a genotype *DCe/DCe* (Fig. 2B). The haplotype *dcE* but not *DCe* of HT had been transmitted to the children (Fig. 2). RT-PCR, exon assay, and sequencing confirmed the assigned genotypes and

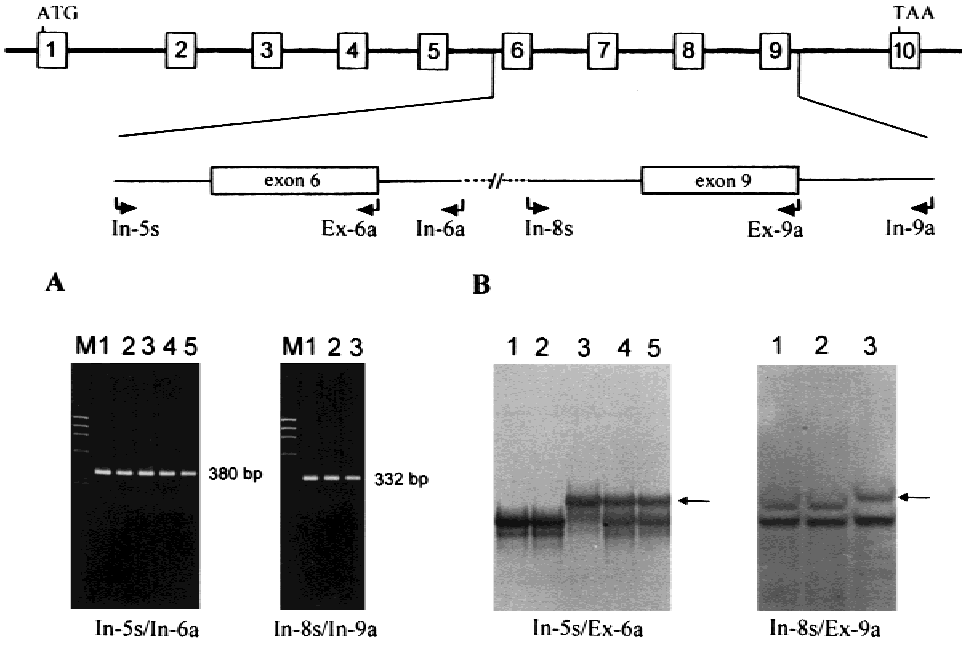

Fig. 4. Amplification and SSCP analysis of the genomic region containing the missense mutations. The region encompassing exon 6 or exon 9 of the Rh50 gene is schematically shown. The sequence was amplified in two segments and denoted by the primers. A: 1.8% agarose gel electrophoresis of the amplified fragments. Left, In-5s/In-6a (380-bp) fragments for exon 6. Lane designations are: 1, D+; 2, D-; 3, Rh_{null}(HT); 4, D+(RT); and 5, D+(YT). Right, In-8s/In-9a (332-bp) fragments for exon 9. Lanes are: 1, D+; 2, D-; and 3, Rh_{null}(WO). B: SSCP analysis of the amplified genomic fragments. Left, In-5s/Ex-6a fragments for exon 6. Right, In-8a/Ex-9a fragments for exon 9. Lanes are the same as in A. The products were separated on 8.0 % polyacrylamide gel electrophoresis followed by silver staining. The shifted bands are denoted by arrows.

showed a normal sequences of Rh transcripts. The results excluded mutation at *RH* and confirmed the occurrence of regulator Rh_{null} in the two cases.

Structure and Expression of Rh50 Gene

Next, we analyzed *RHAG* locus in Rh_{null} cases by Southern blot. An absence of gross change (gels not shown) indicated that some subtle changes might have occurred. Thus, we characterized the Rh50 transcripts expressed in the Rh_{null} cells. As compared with controls, the intensity of Rh50 cDNA bands derived from Rh_{null} probands was not quantitatively different (see below). Sequencing of full-length cDNAs covering the entire coding region of Rh50 identified three nucleotide changes in two exons. In Rh_{null}(HT), two *cis* G→A transitions (nt 808A and 838A) occurred in exon 6 (Fig. 3A). They changed codons GTT to ATT and GGA to AGA, respectively, leading to the Val^{270}→Ile and Gly^{280}→Arg substitutions. The two mutations were cotransmitted from HT to the children (not shown). In Rh_{null}(WO), a single G→T transversion in exon 9 (nt 1139T) was the only defect identified (Fig. 3B). This point mutation converted codon GGT to GTT, resulting in a Gly^{380}→Val change.

Notably, all three missense changes are located in the C-terminal half of Rh50 glycoprotein, with two targeting Gly residues in the TM segments. In Rh_{null} (HT), Val^{270}→Ile was a similar change of aliphatic amino acids located at endoloop 5, whereas Gly^{280} was predicted to reside in the middle of TM9 and was replaced by a positively charged Arg. In Rh_{null}(WO), Gly^{380} is an amino acid close to the C-terminal end of TM12 and was replaced by a hydrophobic residue Val.

Amplification and Analysis of Rh50 Genomic Sequences

To determine the genotype status of the above mutations, we amplified and sequenced the region of *RHAG* spanning exon 6 and exon 9 (Fig. 4A). This verified the result of cDNA analysis (Fig.3), showing that Rh_{null}(HT) and Rh_{null}(WO) were homozygous, while HT's children were heterozygous, for the respective mutations. The observed homozygosity and heterozygosity were further confirmed by SSCP analysis of the fragments containing exons 6 and 9 (Fig. 4B).

Effect of Exon Mutations on Pre-mRNA Splicing

Because 808A and 1139T resided in the +1 position of exons 6 and 9, respectively, we analyzed their potential effect on splicing by rapid amplification of cDNA ends (RACE). The cDNA bands from controls and Rh_{null}(HT) were of identical size and similar intensity (Fig. 5A), suggesting that 808A had no major effect on the splicing of exon 6. In contrast, two bands were seen in Rh_{null} (WO): one comparable to the normal-sized form, and the other as a minor shortened species (Fig. 5A). This result

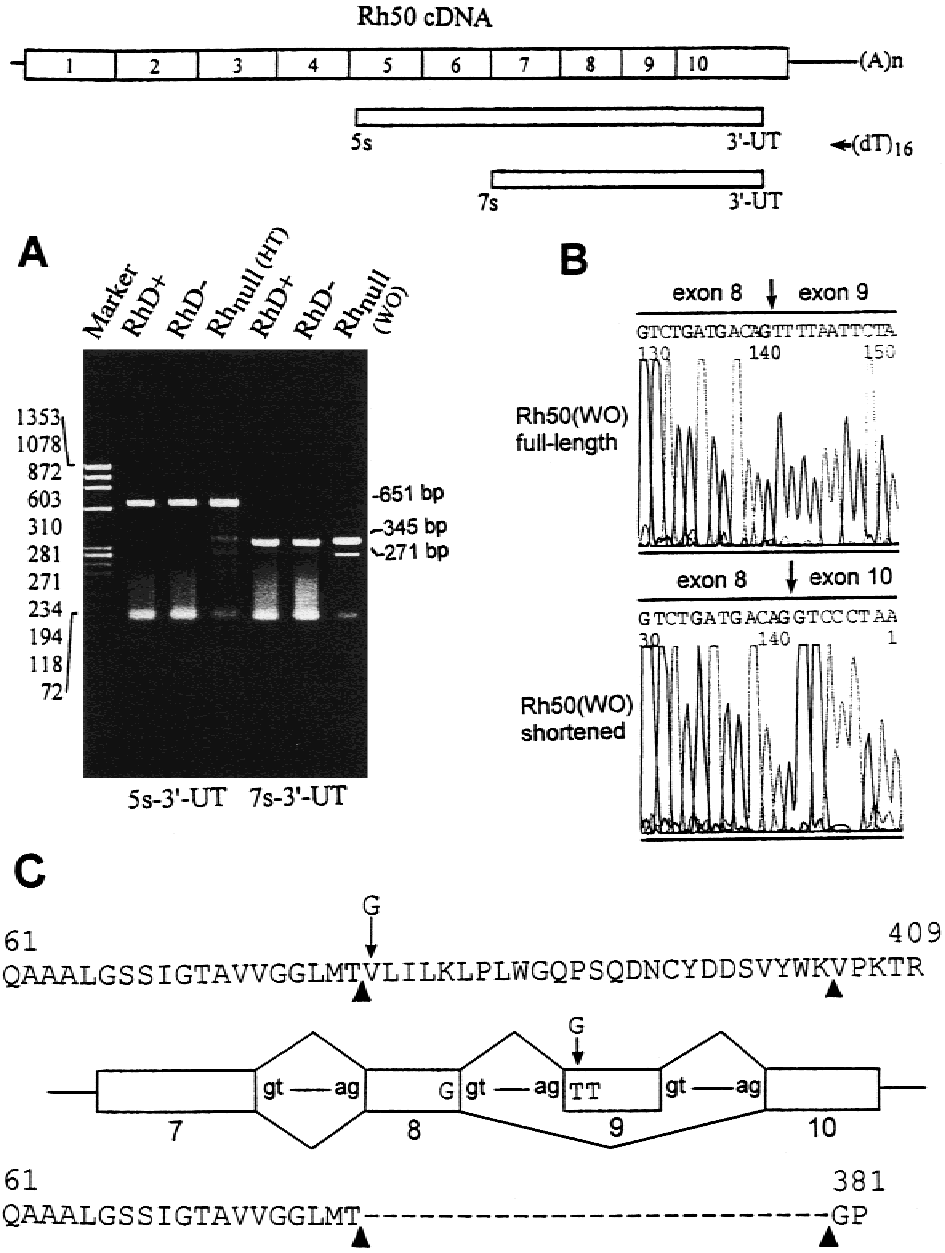

Fig. 5. Effect of exonic 808A and 1139T mutations on pre-mRNA splicing. Strategy for 3′ RACE is diagrammed. cDNA was reverse-transcribed with the $(dT)_{16}$ primer and then amplified with Rh50-specific exon (5s or 7s) and 3′-UT primers. A: Agarose gel electrophoresis of 5s-3′-UT and 7s-3′-UT cDNA segments. The size of *Hae*III-cleaved ϕX174 DNA markers is indicated at left. The size of cDNA bands are indicated at right. Some minor bands seen in Rh_{null} (HT) were nonspecific. In Rh_{null}(WO), a minor band of 271-bp is clearly seen in addition to the expected 345-bp product. B: Sequencing profiles of the Rh50(WO) 345-bp (full-length, upper) and 271-bp (shortened, lower) cDNAs. Arrows denote exon 8/9 and exon 8/10 boundaries. C: Consequence of exon skipping in Rh50(WO). The shortened cDNA is predicted to encode a 381-amino acid polypeptide lacking the cytoplasmic domain. Missense mutations and exon boundaries are illustrated.

suggested that 1139T had caused an aberrant splicing event. Sequencing showed that the normal-sized one retained a connection of exon 8 to exon 9, whereas the shortened one had a ligation of exon 8 with exon 10 because of exon 9 skipping (Fig. 5B). Thus, 1139T was also a splicing mutation leading to a partial inactivation of its adjacent acceptor splice site. Because codon 380 spans both exons 8 and 9 [6], a skipping of the later exon inevitably caused a frameshift and premature chain termination. The minor transcript encodes a 381-amino acid polypeptide lacking the cytoplasmic segment (Fig. 5C). Southern blot analysis of the amplified cDNA also detected other spliced forms of minute amounts in WO and controls (data not shown).

DISCUSSION

We analyzed two Rh_{null} cases at the level of genes, transcripts, and proteins or phenotypes. We identified three new point mutations in their Rh50 gene, but no abnormality in their Rh30 genes. The mutations were shown to target exon 6 or exon 9, causing missense amino acid changes that are localized to the C-terminal region of the Rh50 protein. By genetic definition, these results confirm the linkage of regulator Rh_{null} to *RHAG* in the two probands. As immunoblot analysis showed an apparent absence of both Rh50 and Rh30, the identified molecular defects are likely responsible for the disassembly of the Rh membrane complex under Rh_{null} condi-

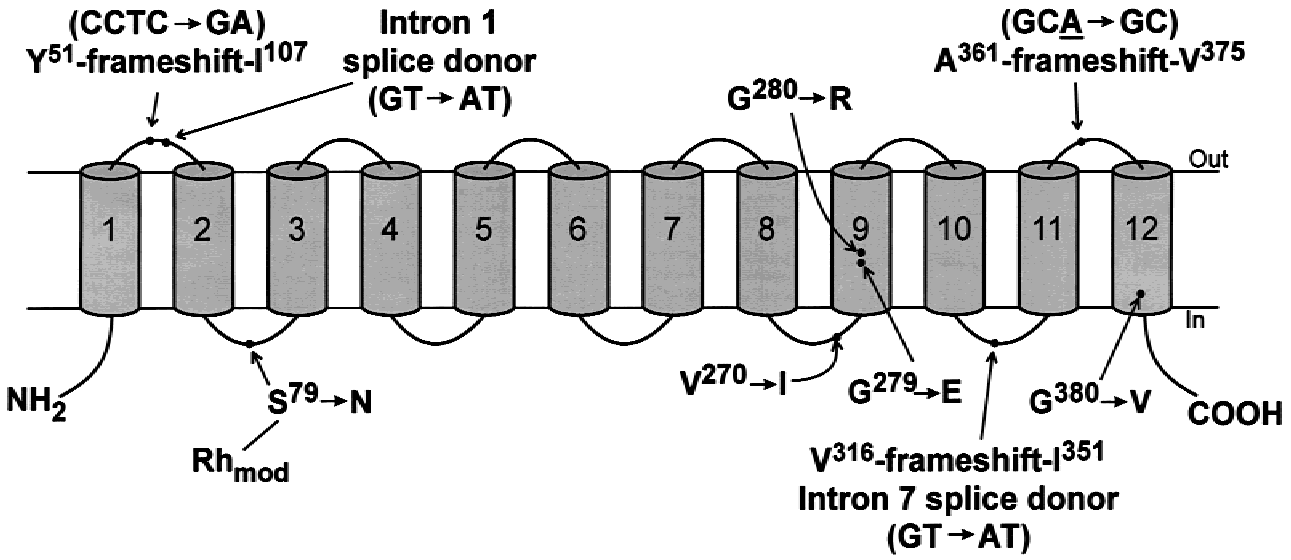

Fig. 6. Schematic of regulator mutations associated with Rh deficiency syndrome. Rh50 is depicted as a 12-TM protein with its N- and C-terminal ends facing the cytoplasm. The numbering of amino acid changes caused by the various mutations are denoted. The small exon deletions (CCTC→GA and GCA→GC) each result in a different frameshift [3]. With regard to the splice donor mutations (GT→AT), the one in intron 7 caused a frameshift [6] and the other in intron 1 completely abolished mRNA splicing and maturation [7]. The predicted beginning and ending of frameshift are indicated by the corresponding amino acids. Of the five missense changes known, Ser79Asn [3] and Val270Ile (this study) are located at the cytoplasmic side, whereas Gly279Glu [7,8], Gly280Arg (this study), and Gly380Val (this study) involve TM segments 9 and 12.

tions. Notably Rh_{null}(HT) is the first case homozygous for two *cis* mutations, differing from another example where a silent splice donor in intron 1 is *trans* to the Gly279Glu missense mutation [7].

Unlike the *RH* locus, the *RHAG* regulator does not exhibit extensive polymorphisms and its identified changes all associate with Rh deficiency syndrome (Fig. 6). Owing to an inherent shift in open reading frame or an inevitable skipping of exons, the exonic deletions or splice site mutations have at large altered the gene expression and/or protein structure [3,6,7]. Nevertheless, with the new mutations described here, five missense changes become known for the Rh50 glycoprotein and yield important information on the molecular basis for the Rh deficiency phenotype. As shown in Fig. 6, two missense changes, Ser79Asn [3] and Val270Ile (this study), face the cytoplasm and three involve Gly residues in TM9 and 12 segments, which include Gly279Glu [7,8], Gly280Arg, and Gly380Val (this study). It should be noted that Ser^{79} and Val^{270} reside in the 1st and 5th endoloops (Fig. 6) and both are variable in homologs from other species [7]. In Rh_{mod}(VL), the Asn^{79} change [3] may affect the interaction of Rh50 with Rh30 [16], but its association with other *cis* or *trans* mutation has yet to be ruled out, as is the relationship of Ile^{270} with Arg^{280} shown for Rh_{null}(HT). Given their C-terminal location, the Gly280Arg or Gly380Val change in Rh_{null}(HT) or Rh_{null}(WO) seem unlikely to exert their effect on translation initiation or termination. With these thoughts in mind, we speculate that the silencing of Rh and related phenotypes stems primarily from some structural alterations and posttranslational defects in the missense proteins themselves.

A survey of 7556 TM segments shows that Gly tends to reside toward the hydrophobic core of TM a-helices [17]. Likewise, replacement of these Gly positions by charged residues with a bulky side chain will not only cause a conformational change but break up the hydrophobic continuity for TM helix formation. This may pertain to the presence of Gly^{280} at the center of TM9. The contrast of Arg^{280} in Rh_{null}(HT) with Glu^{279} in Rh_{null}(YT) [7,8], two adjacent missense mutations of opposite charge, provides compelling examples in regard to the null phenotype (Fig. 6). In both cases, TM9 could be disrupted by the charged residue, which may in turn alter the intramembrane packing of its adjacent TM segments [18] and ultimately affect the interaction of the missense Rh50 with Rh30.

The Gly380Val missense change seen in Rh_{null}(WO) portrays a different mode in altering the TM segment, as no charged residues are involved. Based on hydropathy analysis [9], Gly^{380} is situated at the end of TM12, the last membrane span, and is followed by three bulky, hydrophobic residues, Leu-Ile-Leu. Because of its ability to increase main chain flexibility, Gly^{380} may assume a critical role in the orientation of the cytoplasmic domain and in the interaction of TM12 with the neighboring TM segment. Although Val^{380} is hydrophobic in nature, its

larger side chain may destroy the kink normally conferred by Gly^{380}. This could introduce some local rigidity into the TM α-helix turn, thereby affecting the fold and orientation of TM12 in the Rh50 glycoprotein. The clustered location of mutations (four missense changes, one splice site mutation, and one single nucleotide deletion; Fig. 6) argues for a role of the C-terminal region of Rh50, either directly or indirectly, in the interaction with the Rh30 polypeptides.

Notably, both the 808A transition and 1139T transversion occur in the +1 position of exons 6 and 9 [6]. We examined their potential effect on RNA splicing, as exon sequences, particularly those adjacent to the invariant GT donor and AG acceptor, are important for splice site selection [19]. We showed that 808A did not affect splicing, conforming to the observation that G and A are two favored nucleotides at +1 position in the acceptor splice junction [19]. However, 1139T caused an aberrant splicing due to a partial inactivation of the acceptor splice site. Although the product of exon 9 skipping was not quantified, its presence as a minor species is consistent with the statistic evaluation that the T residue is least frequent at the +1 position of intron/exon junctions [20].

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